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(6) METABOLISM OF FATTY ACIDS AND RELATED SUBSTANCES IN ANIMALS EXPOSED TO COLD,

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METABOLISM OF FATTY ACIDS AND RELATED SUBSTANCES IN ANIMALS EXPOSED TO COLD*

David Rapport
Tufts University School of Medicine
Boston, Massachusetts

Fatty Acid Synthesis by Rat Liver Supernatant with Malonyl-2-C^{14}-CoA as Substrate

The following method was developed for the study of long-chain fatty acid synthesis from malonyl-CoA in the 105,000 x g supernatant prepared from rat liver. The contents of the incubation flask are as follows: 0.5 μmoles acetyl-CoA, 1 μmole malonyl-2-C^{14}-CoA, 48 μmoles KCl, 0.6 μmole $MnCl_2$, 1 μmole potassium succinate, 18.8 μmoles isocitrate, 1.6 μmoles TPN, 4.8 μmoles potassium phosphate buffer, 1.2 μmoles DPN, 8 μmoles potassium becarbonate and 0.1 ml of 20% rat liver supernatant. The pH of the system was around 7.0. The total volume of the system was 1 ml, and it was incubated without shaking in air at 37.5° C for either 30 or 60 minutes. The reaction was stopped by adding 1 ml of ethanol containing 2.5 mg of palmitic acid and 200 mg of KOH. It was heated in a boiling water bath for 30 minutes, then cooled and acidified with 1 ml of 5N HCl. The system was then shaken vigorously in a stoppered tube with 5 ml of hexane for 10 minutes. The hexane was then removed by syringe and put into a graduated tube. This extraction was repeated three times, and the pooled extract was brought to a 20 ml volume in a graduated tube. About 5 ml of the hexane extract was shaken with about 5 ml of water, and then 1 ml of the hexane was dried under infrared heat and counted by the usual manner of this laboratory. This method gave lipogenic activities at least as high as those described previously by us for the acetate system.

Fatty Acid Synthesis by Rat Liver Homogenates with Acetyl-CoA as Substrate

A method of preparing liver supernatant systems capable of synthesizing long-chain fatty acids from acetyl-CoA was developed. The following is a description of the incubation composition: about 0.85 μmoles acetyl-1-C^{14}-CoA, 48 μmoles KCl, 0.6 μmoles $MnCl_2$, 1.0 μmoles potassium succinate, 40 μmoles isocitrate, 2 μmoles TPN, 4.8 μmoles potassium phosphate buffer, 1.2 μmoles DPN, 12 μmoles potassium bicarbonate, 1.5 μmoles ATP, and 0.3 ml of a 20% rat liver supernatant prepared by centrifuging an homogenate at 105,000 x g for 60 minutes. The pH of the system was about 7.0. The final volume of the system was 1 ml and was incubated at 37.5° C for 60 minutes in an atmosphere of 5% Co_2 and 95% O_2. The fatty acids were extracted from the system as described above (the malonyl-CoA system). It was found that lipogenesis from acetyl-CoA was at about the same rate as was found previously from acetate.

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* Studies covering period from 1 February through 30 April 1961. (9) Dept

Assay of the ATPase Activity of Microsomes

We have developed a method for the assay of the ATPase activity of liver microsomes. The following is the composition of the assay mix: 0.75 ml ATP (20 mM) pH 7, 0.75 ml magnesium chloride (20 mM), 0.50 ml tris buffer (0.6 M pH 7.5, 0.25 ml cysteine (1 M) pH 7.5, 0.20 ml 10% microsomes. The final volume of the system was 3 ml and it was preincubated 5 minutes before the reaction was started by the addition of the microsomes. The system was then incubated for 30 minutes at 37° C. The reaction was stopped by the addition of 15% trichloroacetic acid and was centrifuged for 10 minutes. From the supernatant a 1 ml aliquot was taken for inorganic phosphate determination by the method described for the inorganic pyrophosphatase assay.

RESULTS

Further Studies on the Inhibitor of Lipogenesis Induced by Fasting

Considerable progress has been made in the last three months in unravelling the complexities of the lipogenic inhibitor found in rats fasting at either 0° C or 25° C. Since these results depend on a knowledge of information presented in previous reports, it might be wise to review these data briefly in the first two tables. In Table I effects on lipogenesis by homogenates and 105,000 x g supernatant prepared from fed and fasted rats are presented. It can be seen that the homogenate of the fed animal synthesizes far more fatty acids than that of the fasted one, but that the removal of the cytoplasmic particles yields a supernatant which in the case of the fed animal has considerably less lipogenic activity than the homogenate. The opposite result is found with fasting rats, the supernatant being more active than the homogenate.

TABLE I

Physiol. State	mμmoles Acetate-1-C^{14} Conv. to Fatty Acids	
	Homogenate	Supernatant
Fed	354	240
Fasted	93	147

It therefore appeared that in the case of the fasted animal, and the cold-fasted as well, there is an inhibitor of lipogenesis in the particulate fractions of the cell

In order to get definite information about the inhibitor, we studied the effect of adding various cytoplasmic fractions from both fed and fasted animals to the supernatant of the fed animal. The results of this experiment are recorded in Table II.

TABLE II

Liver System	
Fed Supernatant	296
Fed Supernatant and Fed Cytoplasmic Particles	382
" " and Fasted Cytoplasmic Particles	169
" " and Fed Mitochondria	172
" " and Fasted Mitochondria	108
" " and Fed Microsomes	222
" " and Fasted Microsomes	51

The salient features here are that the cytoplasmic particles (i. e., the mixtur of the microsomes and mitochondria) of fed animals greatly promote lipogenesis by the supernatant from fed animals. On the contrary, adding the cytoplasmic particles of fasting animals greatly reduces the lipogenic activity of the supernatant of fed animals. Mitochondria from both fed and fasted animals suppresse lipogenesis in the supernatant to some extent, the fasted somewhat more than in the fed. Microsomes from fed animals are only slightly inhibitory, but microsomes from fasted animals are the most inhibitory particles, the significance being very high when statistically compared to any other particle type. It therefore seemed that the bulk of the inhibitory activity of the liver of fasted animals resides in the microsomes. For this reason we have concentrated our work in the last three months on the microsomes from fasted animals. In Table III are seen the results of studying the effect of the concentration of fasted microsomes on the lipogenic activity of the fed supernatant. The more microsomes present, the greater the inhibition--thus following an expected curve for the presence of an inhibitor.

TABLE III

Conc. of Fasting Microsomes (Expressed as Ratio of Conc. in 20% Homogenate)	Inhibition of Fed Supernatant (%)
0. 50	40
0. 625	47
0. 85	55
1. 00	61
1. 35	67

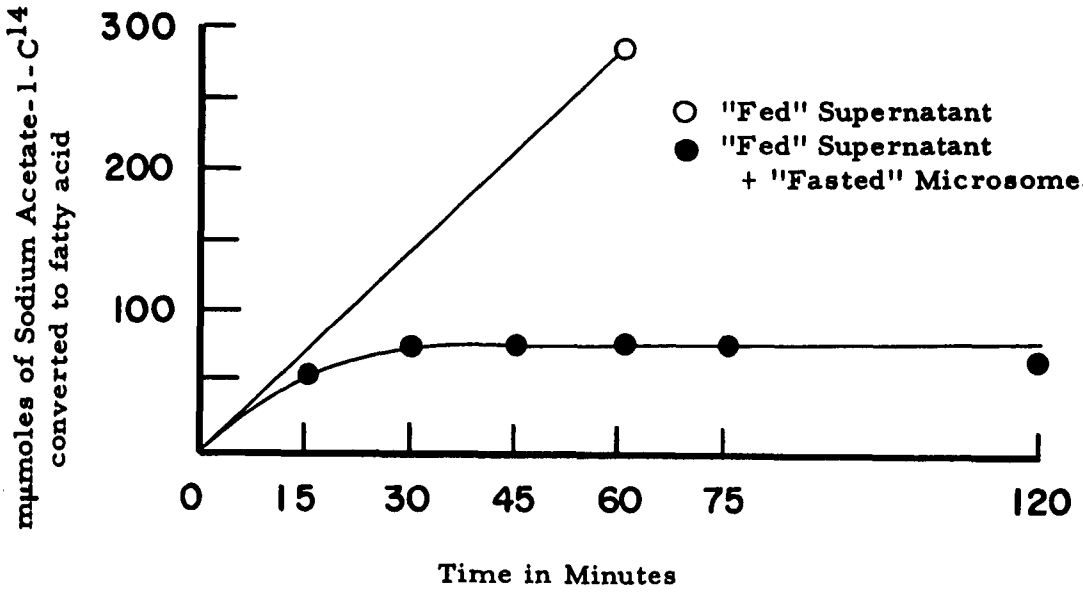
In the last report we stated that fasted microsomes treated with sonic vibration released the inhibitor in a water-soluble form. We also presented evidence that this inhibitor was a protein. During the past three months we have made some attempt to purify this protein further. Its behavior, however, on DEAE-cellulose columns has been very erratic. Reproducibility has been wanting, and evidence for wide distribution of the inhibitory material over various column fractions has been continually found. For this reason we decided to change our approach and get further insight into this inhibitor by means of more biological experimentation. The results of the following experiments indicate considerable success with this approach. In Fig. 1 the time curve of supernatant lipogenesis in the presence of microsomes from fasting animals is plotted. We have previously found that the fed supernatant is perfectly linear with time up to about 2 hours. Therefore in these experiments only the one hour time interval was done for the supernatant without fasted microsomes.

The inhibitor does not immediately stop the lipogenic activity of the fed supernatant, which occurs at a reasonably normal rate for the first 15 minutes. Complete block of the lipogenic activity occurs after this time. Thus it would appear that the inhibitor is destroying some component of the system during the time interval of incubation. As can be seen from Fig. 2, the phenomenon that we see here with fasting microsomes is equally well seen if we use the inhibitor from fasting microsomes obtained by sonic treatment of the microsomes. Again it is the rapid stoppage of fatty acid synthesis after some time that makes clear the difference between lipogenesis with and without the presence of the inhibitor.

We therefore decided to approach this problem by pre-incubation of various combinations in order to see the site at which the inhibitor of lipogenesis is acting. Table IV contains a summary of these data, and as one reads down Table IV one sees that the fed supernatant has a slightly greater rate of lipogenesis if it is pre-incubated for 1/2 hour before the incubation medium containing the labeled acetate is added. The third and fourth lines in the table record the effect of fasted microsomes on fed supernatant with and without pre-incubation, and the usual inhibition is found, the pre-incubation of microsomes alone not affecting the result. The data in the fifth line of the table show that when fasted microsomes are pre-incubated for 1/2 hour with the incubation medium lipogenesis is almost completely abolished. It is also striking, however, that pre-incubation of the fed supernatant with fasted microsomes for 1/2 hour leads also to a very much more severe inhibition of fatty acid synthesis in the fed supernatant system than when no pre-incubation is used. The conclusions that we must draw from this are:

1) that an inhibitor from the microsomes acts on some incubation medium component and apparently destroys it, thereby inhibiting lipogenesis. Of the components of the incubation medium, the ones known to be critical to the rate of lipogenesis are the TPN, isocitrate and ATP. It would seem likely that the lipogenic inhibitor of the fasted microsomes is destroying one or more of these three components of the medium.

— FIGURE 1 —


mμmoles of Sodium Acetate-1-C14 converted to fatty acid
300
200
100
0
15
30
45
60
75
120
Time in Minutes
"Fed" Supernatant
"Fed" Supernatant
+ "Fasted" Microsomes

— FIGURE 2 —

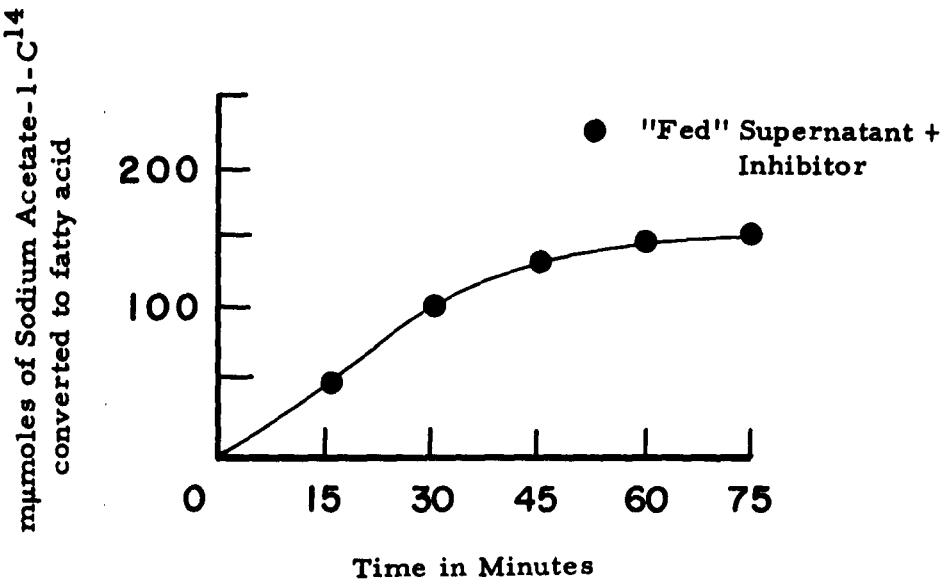

mμmoles of Sodium Acetate-1-C14 converted to fatty acid
200
100
0
15
30
45
60
75
Time in Minutes
"Fed" Supernatant + Inhibitor

TABLE IV

Liver Preparation and Pre-incubation	mμmoles Acetate-1-C^{14} Conv. to Fatty Acids
1) Fed Supernatant	280
2) " " Pre-incubated 1/2 hr.	310
3) " " and Fasted Microsomes	110
4) " " and Fasted Microsomes (Pre-incubated 1/2 hr.)	120
5) " " and Fasted Microsomes (Pre-incubated 1/2 hr. with medium)	10
6) " " and Fasted Microsomes (Pre-incubated 1/2 hr. with fed supernatant)	45

It is also quite clear that there is another inhibitor which must have a completely different action since the pre-incubation with the fed supernatant alone should not cause a limitation of cofactors. Therefore the greater inhibitory action by pre-incubation of fed supernatant with fasted microsomes must be due to a direct attack on the enzyme systems of the fed supernatant. It is therefore likely that the fasted microsomes also contain a lipogenic inhibitor that acts on the lipogenic enzyme system.

In Table V experiments seen in Table IV are repeated with the material liberated by sonicating the fasted microsomes, rather than the microsomes themselves

TABLE V

Liver Preparation and Pre-incubation	mμmoles Acetate-1-C^{14} Conv. to Fatty Acids
Fed Supernatant	220
Fed Supernatant and Soluble Inhibitor	145
" " and Soluble Inhibitor (pre-incubated 1/2 hr.)	155
" " and Soluble Inhibitor (and medium pre-incubated 1/2 hr.)	70

This information can be summed up as follows: The inhibitor that acts on the medium is most certainly released by the sonication of the microsomes. It would appear from these data that the other inhibitor that acts on the enzyme system of the fasted animal is not released; however, before this statement can be definitely made, a further investigation of the phenomenon should be undertaken. To get a further insight on the biochemical site of this inhibition, it was decided to study lipogenesis from acetyl-CoA and malonyl-CoA rather than from acetate. In Table VI it can be seen that the fasted microsomes rather markedly inhibited fatty acid synthesis from acetyl-CoA. Hence at least part of the inhibition of lipogenesis from acetate occurred subsequent to the generation of this substance.

TABLE VI

Liver Preparation	mμmoles Acetyl-1-CoA Conv. to Fatty Acids
Fed supernatant	39
" " and fasted microsomes	13

In Table VII similar experiments were done with labeled malonyl-CoA and it can be seen that there was no inhibitory action at all.

TABLE VII

Liver Preparation	Incubation Time (Minutes)	mμmoles of Malonyl-2-CoA Converted to Fatty Acids
Fed Supernatant	30	39
Fed Supernatant and Fasted Microsomes	30	40
Fed Supernatant	60	63
Fed Supernatant and Fasted Microsomes	60	63

It therefore must be concluded that the enzyme systems subsequent to the generation of malonyl-CoA are not regulated by either of the inhibitors. In considering the cofactors that would not be needed subsequent to malonyl-CoA formation, it was at once evident that of the three named above, ATP was the one. It therefore seemed likely that the inhibitor acting on the incubation medium is an ATPase. To test this further, we decided to measure directly the ATPase activity of the normal fed microsomes and of the fasted microsomes. The results of this work are recorded in Table VIII.

TABLE VIII

Physiol. State	mμmoles Pi Liberated/mg Protein
Fed	3.60
Fasted	7.03

Clearly the ATPase activity in the microsomes of fasted rats is much higher than in the fed rats. It would seem that the concept that the inhibitor is an ATPase, or at least that one of the inhibitors is, is supported by the above data. In conclusion, therefore, we find that there are at least two lipogenic inhibitors in fasting microsomes. One of these inhibitors acts on the cofactors of the system and appears to be an ATPase. The other attacks the enzyme system itself, and as of yet no work has been done on its mechanism of action. It is hoped in the next three months we will extend the investigation into a detailed analysis of the latter inhibitor.

Effects of Cold Acclimation on Hepatic Vitamin A Content

Some time ago we reported that the vitamin A content of the cold-acclimated rat was considerably above that of the rat fed the same diet at 25° C. These data are reviewed in Table IX.

TABLE IX

Physiol. State	No. of Rats Used	Vitamin A Content of Liver	
		Total γ	γ/g wet wt.
Acclimated to 25° C	6	12,230 ± 2066*	1160 ± 73*
Acclimated to 0° to 2°C	6	24,400 ± 5660	2950 ± 361

* Standard Error of the Mean.

These findings are quite interesting because in 1943 Rodahl and Moore reported that the liver of the polar bear contains a very large amount of vitamin A. These authors concluded that the toxic effects following the ingestion of polar bear liver are symptoms of hypervitaminosis A. They also mentioned that similar findings are sometimes seen with the liver of seals. It would seem most likely that this high hepatic vitamin A content of the polar bear and seal liver is related to the nature of their diet. However, it seems also possible that low environmental temperature may play a role. To test this possibility further, we carried out experiments in which the vitamin A intake of the animal was carefully controlled and was the same for both the animals living at 25° C and the cold-acclimated animals living at 0° to 2° C. The rats were given a dose of vitamin A just above the minimum requirement for a rat (i. e., 40 units vitamin A/week). It was felt that this low dosage would be best for bringing out any change in vitamin A metabolism in cold-acclimated animals, leading to an action that spares vitamin A. The results of these experiments are recorded in Table X.

TABLE X

Physiol. State	No. of Rats Used	Vitamin A Content of Liver	
		Total γ	γ/g wet weight
Acclimated to 25° C	9	103 ± 10.4	10.2 ± 1.13
Acclimated to 0° to 2°C	10	57 ± 7.8	6.5 ± 0.85

When fed the same amount of vitamin A the cold-acclimated rats stored a little less vitamin A in the liver than did the rats acclimated to 25° C, the p value being less than 0.01 when expressed on the basis of total liver, and 0.02 when expressed on the basis of per gram liver weight. It is clear that, in the case of rats fed a vitamin A-containing diet ad libitum, the increase in vitamin A content induced by cold acclimation results from an increase in food consumption. No evidence was found to indicate that cold acclimation acts to spare vitamin A. It is difficult to know to what extent the natural exposure of polar bears or seals to low environmental temperatures is responsible for the accumulation of vitamin A in their livers. However, it would seem probable that the eating response of most mammals when exposed to low temperatures would lead to an increase in hepatic vitamin A content. The data presented above allow one to draw some conclusions regarding the effects of cold acclimation on vitamin A requirements of rats. It is a striking fact that, on a vitamin A intake close to the minimum requirements of rats living at ordinary room temperature, the cold-acclimated rat gets along very well as judged by appearance, survival and body weight. It is true that the cold-acclimated rats with the low vitamin A intake stored somewhat less vitamin A in their livers than did similarly treated rats living at 25° C, but the difference is surprisingly small when it is considered

that four months of living is involved. It is evident that the intake of vitamin A need not parallel the increase in food consumption for the rat to cope quite successfully with low environmental temperatures.

Fatty Acid Synthesis from Glucose Administered by Stomach Tube to Control and Cold Acclimated Rats

In order to extend the liver slice work and adipose tissue work to their physiologic meaning in the intact animal, studies were carried out in which one gram of labeled glucose was administered to rats that had been fasted for 3 hours. The rats were then allowed to eat the standard diet and 6 or 24 hours later were sacrificed. The total incorporation of glucose-C^{14} into fatty acids was measured both for the whole animal and for various tissue sites such as liver and adipose tissue. As yet we have not done enough animals to discuss the possible differences in lipogenesis between control and cold-acclimatized animals. However, some rather unexpected findings were found which bear mentioning at this time. In Table XI the specific activities of the fatty acids isolated from the various tissues studied, as well as the whole animal, are recorded. These results are for the 6-hour time period.

TABLE XI

Tissues	Specific Activity cts/min/mg/Fatty Acids	
	For Control	For Cold-Acclim.
Liver	422	139
Epididymal Fat Pad	4	2
Mesenteric Adipose Tissue	45	9
Lumbar Adipose Tissue	72	10
Cutaneous Adipose Tissue	10	.2
Interscapular Brown Adipose Tissue	3820	710
Brain	54	136
Blood	42	53
Gut	468	137
Carcass	161	50
Whole Rat	165	43

It will be seen from this that, with the exception of the brown adipose tissue in the interscapular area, all other adipose tissue sites have a lower specific activity than the whole rat or the rat carcass. This finding is unexpected since it is

widely believed that the adipose tissue of rats is primarily, if not almost exclusively, responsible for their fatty acid synthetic activity. The above data make it difficult to believe that such is the case. However it is conceivable that, if the adipose tissue regions of the body have fatty acid pools which are specifically involved in the fatty acid synthetic process, and if, further, these pools--after synthesizing the fatty acid--store reasonably small quantities, releasing the rest to the body in general, then our data may not be incompatible with the adipose tissue being the major site of fat synthesis. In the near future we hope to gain further insight into this possibility by separating the cytoplasm from the bulk fat of the adipose tissue. It is reasonable to assume that the cytoplasm is the only area of fat synthesis in the adipose tissue, and therefore the specific activities in this region should be very high indeed if the adipose tissue is primarily or exclusively involved in the synthesis of long-chain fatty acids. The very high specific activity found in the interscapular brown adipose tissue may be of considerable significance in the cold-acclimated rat, since these rats have rather large amounts of brown adipose tissue not only in the interscapular region but at the borders of the lumbar adipose tissue and around the major arteries. We intend to explore this possibility further also. It can be seen here that, from the appearance of our data, the liver and the gut are the most active sites of fat synthesis; this of course confirms the many workers who have found high specific activities in these regions. The brain gave very surprising results, since these are adult animals. It has been generally claimed that the adult brain does not synthesize long-chain fatty acids. As can be seen here, the brain was very active in converting glucose to fatty acids. It is impossible to believe that the labeled fatty acids in the brain got there by transport, since it is rather well-established that the transport forms of the various fatty acids are not capable of penetrating the blood-brain barrier. We intend to do considerably more work on the physiologic meaning of synthesis of fatty acids by brain in an effort to explain the discrepancy of our work with that of the field in general.